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Histone methylation controls telomerase-independent telomere lengthening in cells undergoing dedifferentiation

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Abstract

Cellular dedifferentiation underlies topical issues in biology such as regeneration and nuclear cloning and has common features in plants and animals. In plants, this process characterizes the transition of differentiated leaf cells to protoplasts (plant cells devoid of cell walls) and is accompanied by global chromatin reorganization associated with reprogramming of gene expression. A screen for mutants defective in proliferation and callus formation identified *kyp-2*—a mutant in the *KRYPTONITE* (*KYP*)/SUVH4 gene encoding a histone H3 lysine 9 (H3K9) methyltransferase. Analysis of telomere length revealed stochastic telomerase-independent lengthening of telomeres in wild type but not in *kyp-2* protoplasts. In *kyp-2* mutant, telomeric repeats were no longer associated with dimethylated H3K9. The Arabidopsis *telomerase reverse transcriptase* (*tert*) mutant displayed accelerated proliferation despite its short telomeres, though it also showed accelerated cell death. Microarray analysis uncovered several components of the ubiquitin proteolytic system, which are downregulated in *kyp-2* compared to wild-type protoplasts. Thus, our results suggest that histone methylation activity is required for the establishment/maintenance of the dedifferentiated state and/or reentry into the cell cycle, at least partly, through activation of genes whose products are involved in the ubiquitin proteolytic pathway. In addition, our results illuminate the complexity of cellular dedifferentiation, particularly the occurrence of DNA recombination that can lead to genome instability.

Keywords: Dedifferentiation; Pluripotency; Histone methylation; Chromatin; Telomeres; Alternative lengthening of telomeres (ALT); Proliferation; Callus; Protoplasts; Arabidopsis

Introduction

Cellular dedifferentiation underlies topical issues in biology such as regeneration and nuclear cloning and has common features in plants and animals (Echeverri and Tanaka, 2002; Wilmut et al., 2002; Odelberg, 2002; Grafi, 2004; Brockes and Kumar, 2005). Dedifferentiation signifies the withdrawal of cells from a given differentiated state into a 'stem cell'-like state that confers pluripotency—a process preceding reentry into the cell cycle (reviewed in Grafi, 2004). This early phase has been hardly addressed, largely because of lack of a suitable experimental system. This limitation is easily overcome by the use of the plant protoplast system (Fig. 1A). Treatment of differentiated mesophyll cells with cell-wall-degrading enzymes generates a large population of protoplasts (plant cells devoid of cell walls), which in the course of their formation have acquired a state of dedifferentiation but are not yet committed to any specific fate. The state of dedifferentiation is evidenced by changes in cell morphology, genome organization and the pattern of gene expression, as well as by the capability of protoplasts to differentiate into multiple types of cells depending on the type of stimulus applied (Takebe et al., 1971; Valente et al., 1998; Zhao et al., 2001; Avivi et al., 2004). Dediffe-

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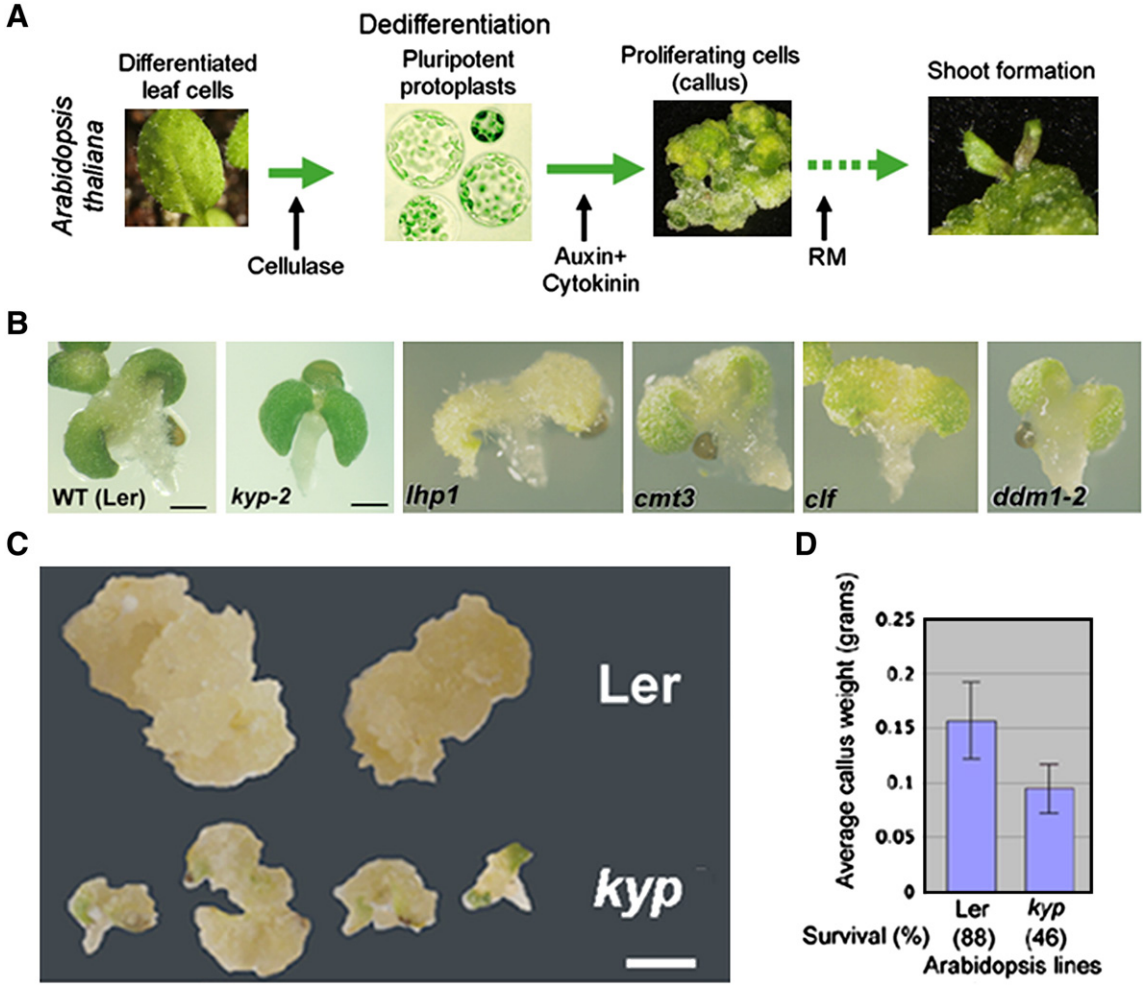

Fig. 1. Screening for mutants in chromatin modifying genes impaired in callus formation. (A) The *Arabidopsis* protoplast system for studying cellular dedifferentiation. Differentiated leaf cells respond to the removal of cell wall (cellulase) by undergoing dedifferentiation and forming pluripotent protoplasts (Grafi, 2004). At this stage, additional signals determine cell fate. Application of auxin and cytokinin induce reentry into the cell cycle, proliferation and formation of callus from which, under appropriate regeneration medium (RM), shoots and roots can be regenerated to form the entire fertile plant (Takebe et al., 1971). (B) Impaired callus formation in the *kyp-2* mutant, carrying a mutation in the *Kryptonite* gene encoding a lysine 9 histone H3 methyltransferase. Images were taken after 14 days on callus inducing medium (CIM). Ler, wild-type *Arabidopsis* ecotype Landsberg erecta; *lhp1*, *like heterochromatin protein 1*; *cmt3*, *chromomethylase3*; *clf, curly leaf*; *ddm1-2*, *decrease in DNA methylation1*. Bar=2 mm. (C) Callus formation in *kyp-2* mutant compared to wild-type (WT, Ler) plants after 24 days on CIM. Each callus developed from a single seed. Bar=3 mm. (D) Reduced leaf explants survival (%) and callus weight (after 28 days on CIM) in *kyp-2* relative to wild type (ecotype Ler). Bars represent the standard deviation.

rentiation was shown to be accompanied by global chromatin reorganization (Blank et al., 1992; Zhao et al., 2001; Williams et al., 2003; Avivi et al., 2004) associated with changes in patterns of gene expression (Jamet et al., 1990; Criqui et al., 1992; Avivi et al., 2004). Chromatin reorganization appears to be a fundamental theme in cellular dedifferentiation and reentry into the cell cycle both in plants and animals (Chiabrera et al., 1979; Blank et al., 1992; Kikyo et al., 2000; Gonda et al., 2003). Thus, studying dedifferentiation in plants might have bearing on various dedifferentiation-driven cellular processes in animals such as regeneration and nuclear cloning.

The basic structural unit of chromatin is the nucleosome, which is composed of DNA wrapped around histone octamer containing two copies of each of the four core histone proteins, H2A, H2B, H3 and H4 (Kornberg and Lorch, 1999); additional factors are involved in the organization of this basic chromatin unit into higher order chromatin structure (Grewal and Moazed, 2003). The 'histone code' hypothesis suggests that chemical modifications of the DNA (e.g., cytosine methylation) or of histone proteins (e.g., acetylation, methylation and phosphorylation) (Wolffe, 1992) generate 'codes' for the recruitment of proteins or protein complexes that affect chromatin structure and gene expression (Jenuwein and Allis, 2001; Zhang and Reinberg, 2001). For example, methylation of cytosine at CpG context generates a binding site for methyl-CpG binding domain (MBD) proteins (Zemach et al., 2005; Scebba et al., 2003; Hendrich and Tweedie, 2003), while methylation of histone H3 at lysine 9 residue by specific methyltransferases [e.g., SUV39H1 in humans (Rea et al., 2000) and KYP/SUVH4 in *Arabidopsis* (Jackson et al., 2002; Malagnac et al., 2002) generates a 'code' for the recruitment of HP1 proteins (Bannister et al., 2001; Lachner

et al., 2001) leading to the formation of compact chromatin structure.

To assess the importance of chromatin remodeling for cellular dedifferentiation and proliferation, we screened *Arabidopsis* mutants in chromatin modifying genes for their ability to form callus when triggered by callus inducing media (CIM). Out of several mutants examined, only *kyp-2* carrying a mutation in the gene *KYP/SUVH4* encoding a histone H3 lysine 9 methyltransferase (H3K9 HMTase) was impaired in callus formation. Further analysis showed the involvement of KYP/SUVH4 in telomerase-independent telomere lengthening during dedifferentiation, as well as in transcription of certain genes involved in cell cycle progression.

Materials and methods

Plant growth, callus formation, protoplast isolation, DNA extraction and southern blot analysis

Seeds of *Arabidopsis thaliana* (ecotypes Colombia and Landsberg erecta, Ler), *kyp-2*, *cmt3* (kindly provided by S. Jacobsen), *ddm1-2* (kindly provided by E. Richards), *lhp1* (kindly provided by V. Gaudin) and *clf* (kindly provided by J. Goodrich) were sown and incubated at 4 °C for 4 days and then grown under short day condition (8 h light) at 21 °C. For callus formation assay, plants were grown aseptically on solid Murashige-Skoog (MS, Duchefa) containing 2% sucrose under long day photoperiod at 22±2 °C. Leaf explants (derived from 18-day-old plants) or sterilized seeds (in 70% ethanol) were placed on callus inducing medium [CIM, MS supplemented B5 salts, 3% sucrose, 2.2 mg/l 2,4-D and 0.05 mg/l kinetin), incubated under the same conditions described above and inspected for callus formation at various time points.

Protoplasts were isolated from rosette leaves of 5–6-week-old plants essentially as described (Zelcer and Galun, 1976), except for cellulase and macerozyme that were supplied at concentrations of 1% and 0.25%, respectively. DNA was extracted from *Arabidopsis* leaves and protoplasts by the C-elyltrimethyl ammonium bromide (CTAB) method. To determine telomere length, genomic DNA was digested with appropriate restriction endonucleases (indicated in figures' legends), separated on 0.8% agarose gel, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled telomeric repeats (pAtT4, a gift from E. Richards) (Richards and Ausubel, 1988) or with ^{32}P-labeled At1g01010 coding sequence. DNA probes were labeled with [α^{32}P]dCTP using the Nick Translation Kit (Roche) according to the manufacturer's protocol.

Protein extraction and telomeric repeat amplified protocol (TRAP)

Proteins for TRAP assay were extracted from *Arabidopsis* essentially as described (Fajkus et al., 1998) and stored at −80 °C until use. Telomerase extension reactions were carried out as described (Kwon and Chung, 2003) in TRAP buffer [20 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, and 1 mM EGTA] containing 200 nM GG primer 5-CACTATC-GACTACGCGATCGG-3′, 100 mM of each dNTP, and 1.5 μg total protein extract. Reaction mixture was incubated at room temperature for 45 min followed by extraction of the extended products with phenol/chloroform and ethanol precipitation. DNA samples were then amplified by PCR using 200 nM GG primer and 200 nM telomeric repeat antisense primer 5′-CCCTAAAC-CCTAAACCCTAAA-3′. PCR was performed for 30 cycles (94 °C/0.5 min, 62 °C/0.5 min, and 72 °C/0.5 min) and culminated with an additional 10 min extension step at 72 °C. PCR products were resolved by 10% nondenaturing polyacrylamide gel stained with SYBR Green (Molecular Probes) and visualized using the Typhoon Laser Scanner (Amersham Biosciences).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed on nuclei prepared from leaves and protoplasts of wild-type (Ler ecotype) and kyp-2 plants essentially as described (Lawrence et al., 2004) using anti-dimethylated K4 histone H3 (Upstate Biotechnology), and anti-dimethylated K9 histone H3 (Upstate Biotechnology). Precipitated DNAs were subjected to dot-blot analysis using ^{32}P-labeled pAtT4 plasmid, or to PCR using the following primers:

CEN180-S 5′-GAGAGGATCCCGTAAGAATTGTATCCTTGTTAG
CEN180-AS 5′-GAGAGAATTCCCTTTAAGATCCGGTTGTGG
Actin-S 5′-GGTTTTGCTGGGGATGATGC
Actin-AS 5′-CATTGAATGTCTCAAACATGATTTGAGTC

ChIP PCR conditions were 94 °C, 5 min; 30 cycles of 94 °C, 30 s; 56 °C, 30 s; 72 °C, 45 s; 72 °C, 10 min. PCR products were resolved on 1.2% agarose gel and stained with ethidium bromide.

Affymetrix microarray analysis

Total RNAs were prepared from wild-type Ler and *kyp-2* protoplasts using RNeasy Plant Mini Kit (Qiagen). Labeled cRNA was prepared and hybridized to Affymetrix ATH1 GeneChips containing 22,810 Arabidopsis genes, according to the manufacturer's guidelines (Affymetrix, Santa Clara, CA). Signal values were obtained using Affymatrix Microarray Suite software version 5.0. Each chip was normalized to its 50th percentile median and multiplied by a factor of 50. Genes with multiple or unambiguous probes were removed. Queries were performed using a custom query interface developed in Microsoft Access. Variation of data between replicates was evaluated, and as all replicates clustered together, further analysis was carried out on mean values. To perform samples clustering, a list of genes differentiating the samples was obtained using ANOVA for all samples in GeneSpring, with FDR of 0.01 and Benjamini and Hochberg correction for multiple testing. Pearson correlation clustering was performed in GeneSpring using this list. Further data filtering and analyses were carried out with Microsoft Excel spreadsheet software.

Results

Kyp-2 mutant is impaired in callus formation

One way to track cellular dedifferentiation in plants is to examine the capability of differentiated cells to reenter the cell cycle, proliferate and form callus. Hence, screening for mutants impaired in callus formation is a sound strategy to pinpoint genes involved in cellular dedifferentiation and proliferation (Konishi and Sugiyama, 2003). We selected several *Arabidopsis* mutants in chromatin modifying genes and examined their capability to form callus when triggered by callus inducing media (CIM). Among the various mutants tested (Fig. 1B), only *kyp-2*, a mutant in the *KRYPTONITE* (*KYP*)/*SUVH4* gene encoding a H3K9 HMTase (Jackson et al., 2002; Malagnac et al., 2002), was significantly impaired in callus formation. After 2 weeks on CIM more than 80% of *kyp-2* seeds failed to form callus compared to 14.5% of wild-type seeds (Supporting data, Table 1). The *kyp-2* mutant displayed a delay in callus formation, reduced callus size, and lower survival of leaf explants compared to wild-type (WT) plants (Figs. 1B–D). Notably, although *kyp-2* mutant shows significant reduction in H3K9 dimethylation (Jasencakova et al., 2003; Jackson et al., 2004), it displays normal growth and development through many generations. In other mutants, callus formation was either accelerated, as in *lhp1,* a mutation in *Like-Heterochromatin Protein 1* gene (Gaudin et al., 2001), or normal as in *ddm1-2*, a mutation in a SWI2/SNF2 gene leading to decreased DNA and histone methylation (Jeddeloh et al., 1999). Normal callus

formation was also found in *cmt3,* a mutation in the *CHROMOMETHYLASE3* gene affecting CpNpG (N = any nucleotide) methylation (Lindroth et al., 2001; Bartee et al., 2001), and *clf*, a mutation in the polycomb *Curly Leaf* gene (Goodrich et al., 1997). The finding that *ddm1-2* was capable to form callus despite a dramatic reduction in H3K9 dimethylation suggests that *de novo* KYP activity is required for normal dedifferentiation and/or proliferation to take place.

Telomerase-independent telomere lengthening during dedifferentiation involves KYP/SUVH4 activity

A characteristic feature of organ regeneration in animals is the length of telomeres (Rudolph et al., 2000; Satyanarayana et al., 2003). Interestingly, cultured embryonic cells of mice carrying mutations in Suv39h1 and Suv39h2, both known to methylate H3-K9 at heterochromatic regions, displayed abnormal increase in telomere length (Garcia-Cao et al., 2004). In view of the linkage between telomere metabolism and H3-K9 methylation, we hypothesized that the observed effect of *KYP/SUVH4* mutation on cell proliferation and callus formation is mediated by telomeres. Using southern blot analysis we followed changes in telomere length during the transition from differentiated leaf cells to protoplasts. Results showed a significant increase in telomere length in protoplasts independently of cellular proliferation (Fig. 2A). This increase in telomere length, however, appeared to be a stochastic phenomenon, that is, in certain experiments telomere lengthening was not apparent (as in Fig. 2D, Ler/EXP2). Telomere lengthening did not result from partial DNA digestion inasmuch as the digestion pattern of a subtelomeric DNA sequence corresponding to the At1g01010 gene was similar in leaves and protoplasts (Fig. 2B). Using the telomere repeat amplification protocol (TRAP) assay, we showed (Fig. 2C) that telomere lengthening was not dependent on telomerase activity; while protein extract derived from dividing cells displayed, as expected, high RNA-dependent telomerase activity, neither leaf cells nor protoplasts showed any apparent activity. Thus, telomere lengthening during dedifferentiation appears to be controlled stochastically by a telomerase-independent mechanism(s) known as alternative lengthening of telomeres (ALT) (Henson et al., 2002). Unlike wild-type protoplasts, *kyp-2* protoplasts displayed no increase in telomere length (Fig. 2D), suggesting that KYP/SUVH4 activity is required for ALT in *A. thaliana*.

Telomeres in Arabidopsis are associated with methylated H3K9

KYP/SUVH4 HMTase in *Arabidopsis* is known to methylate histone H3 mainly at heterochromatic chromocenters (Jasencakova et al., 2003). To further verify the linkage between KYP activity and telomere metabolism, we next studied the association of telomeres with K9-methylated histone H3. We used chromatin immunoprecipitation (ChIP) assay followed either by dot-blot analysis to detect telomeric repeats or by PCR to detect the centromeric 180 bp repeats (CEN180) or actin-encoding sequence. In wild-type (WT) and *kyp-2* plants, the actin-encoding gene was associated with K4—but not with K9-methylated H3. A dramatic reduction in K9-dimethylated H3 associated with CEN180 was observed in *kyp-2* cells (Fig. 3),

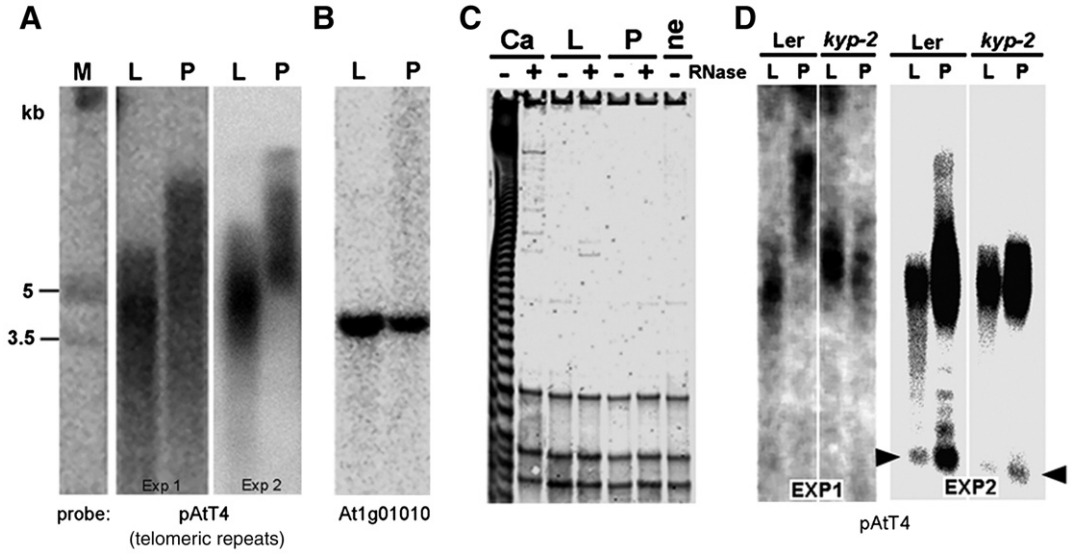

Fig. 2. Telomerase-independent lengthening of telomeres in *Arabidopsis* cells acquiring pluripotency. (A) Lengthening of telomeres in WT Arabidopsis (Columbia ecotype) protoplasts. Genomic DNAs extracted from differentiated leaf cells (L) or pluripotent protoplasts (P) were subjected to digestion with *Eco* RV, followed by southern blotting with ^{32}P-labeled telomeric repeats or with ^{32}P-labeled At1g01010 coding sequence to monitor the extent of genomic DNA digestibility (B). DNA marker sizes (M) are indicated. (C) Leaf cells and pluripotent protoplasts lack telomerase activity. Telomerase activity was assayed using the telomeric repeat amplification protocol (TRAP) with 1.5 μg total protein extract from callus (Ca), leaves (L) and protoplasts (P) in the presence or absence of RNase A. ne, no protein extract. (D) *Kyp-2* protoplasts show no increase in telomere length. DNA was extracted from leaves and protoplasts derived from wild-type (Ler) or *kyp-2* plants, digested with *Dra*I and subjected to southern blot analysis with ^{32}P-labeled telomeric repeats. Arrowheads indicate signals derived from interstitial telomeric repeats (Riha et al., 2001).

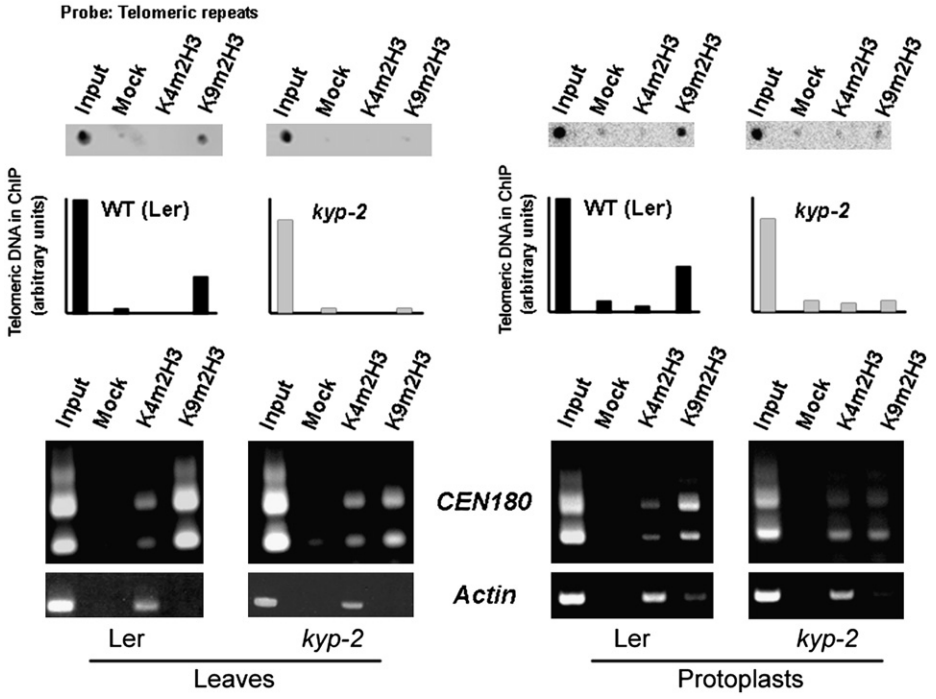

Fig. 3. Arabidopsis telomeres are associated with K9-dimethylated histone H3. Chromatin immunoprecipitation was performed on nuclei from leaves and protoplasts of wild-type Ler and *kyp-2* mutant. Chromatin was immunoprecipitated using anti-dimethylated K4 histone H3 (αK4m2H3), anti-dimethylated K9 histone H3 (αK9m2H3) or no antibody (Mock). DNA was extracted and subjected either to PCR using sets of primers to amplify the centromeric 180 bp repeats (CEN180) or the actin-encoding sequence, or subjected to dot-blot analysis using ^{32}P-labeled pAtT4. Input represents 0.02% of the chromatin subjected to immunoprecipitation.

consistent with a previous report (Johnson et al., 2002). Analysis of telomeres showed that in wild-type leaves, as well as in protoplasts, telomeres are associated with K9—but not with K4-dimethylated H3 (Fig. 3), suggesting that telomeres in *Arabidopsis* have features of compact chromatin structure. In *kyp-2* mutant, telomeres were no longer associated with K9-dimethylated H3, thus pointing to the importance of KYP in regulating telomeric chromatin configuration.

Accelerated cell cycling in tert mutant

To assess the importance of telomere length for cell cycle progression and callus formation, we next examined the capability of Arabidopsis telomerase reverse transcriptase (*tert*) mutant to form callus. It has previously been shown that *Arabidopsis* plants lacking telomerase activity can survive up to 10 generations; late-generation mutants having very short telomeres displayed extended life span and remained metabolically active (Riha et al., 2001). We generated a homozygous *tert* line from a heterozygous population of T-DNA insertion in the single *AtTERT* gene obtained from the Arabidopsis Biological Resource Center (ABRC). This line was established through successive generations of selfing. Southern blot analysis showed (Fig. 4A) that generation 4 (G4) *tert* plants displayed a very short telomere length compared to wild-type plants. Unexpectedly, callus formation analysis showed (Fig. 4B) inverse correlation between telomere length and division rate, that is, G4 *tert* seeds displayed accelerated cell cycling and higher callus weight compared to wild-type plants. However, G4 *tert* derived calluses underwent faster browning and death than wild type calluses (Fig. 4C). This is, probably, due to dramatic loss of telomeric DNA and increased frequency of chromosomal bridges (Fig. 4D), which is characteristic of cells having short telomeres (Zhang et al., 1999; Gisselsson et al., 2005). Thus, the failure to increase telomere length observed in *kyp-2* protoplasts cannot be accounted for the delay in callus formation.

Alteration in gene expression pattern in kyp-2 protoplasts

Another possible model is that KYP HMTase may be required for *de novo* expression of specific genes whose products are involved in setting up the dedifferentiated state or in reentry into the cell cycle. To pinpoint possible factor(s) regulated by KYP HMTase during dedifferentiation we have compared the transcriptome profile of *kyp-2* protoplasts with that of wild-type 'Landsberg erecta' (Ler) protoplasts using the Affymetrix–Arabidopsis ATH1 genome array. We confirmed Affymetrix data by northern blot analysis (Supporting data, Fig. 1). The Affymetrix microarray study revealed that several genes that were upregulated in wild-type protoplasts remained silent or displayed mild activation in *kyp-2* protoplasts (Table 1), and *vice versa,* several genes that were silent in wild-type protoplasts underwent activation in *kyp-2* protoplasts (Supporting data, Table 2). Intriguingly, three genes encoding components of the ubiquitin proteasome system, namely, E2 ubiquitin conjugating enzyme 8 (UBC8/At5g41700), RING-H2 finger-type E3 ubiquitin ligase (At5g37230) and ubiquitin con-

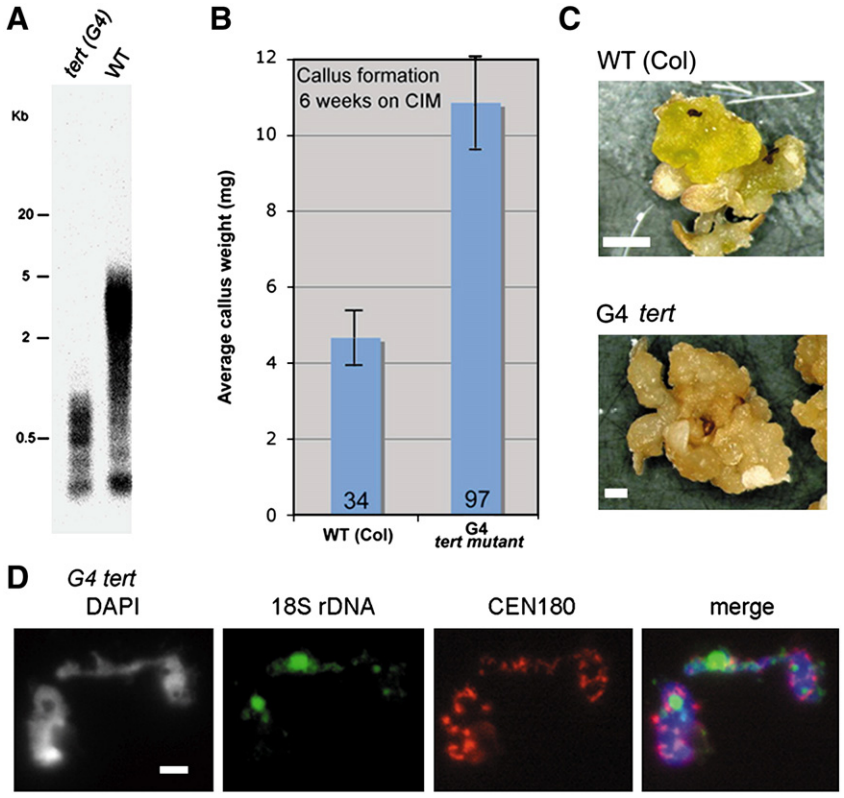

Fig. 4. Accelerated cell division and cell death in *tert* mutant with short telomeres. (A) Generation 4 (G4) *tert* mutant displays a significant shortening of telomeres. Genomic DNAs extracted from G4 *tert* and wild-type (WT) plants were subjected to digestion with *Dra* I followed by southern blotting with ^{32}P-labeled pAtT4. (B) Accelerated callus formation in *tert* G4. Average callus weight±standard deviation was calculated for each line 6 weeks after incubation on CIM. The number of calluses analyzed is indicated at the bottom of each column. (C) Accelerated browning and death of *tert* G4 calluses. Note the green color of the wild-type callus compared to *tert* G4. Bar=5 mm. (D) Chromosome bridges in G4 *tert* cells. Fluorescence in situ hybridization was performed with rhodamine-labeled 180 bp centromeric repeats (CEN180 in red) and fluorescein-18S rDNA (green). DAPI was used as a counter-stain.

jugating enzyme (UBC34/At1g17280) were downregulated in *kyp-2* compared to wild-type protoplasts.

Discussion

We demonstrated here the importance of the chromatin modifying factor KYP/SUVH4 HMTase in regulating cellular dedifferentiation and/or cell proliferation. Histone methylation appears to be a key element regulating both epigenetic reprogramming of gene expression as well as genetic variation driven by DNA recombination as demonstrated by the telomerase-independent telomere lengthening. Thus, the increase in telomere length in the course of cellular dedifferentiation might be controlled by a mechanism, which is known as alternative lengthening of telomeres (ALT) (Henson et al., 2002). Mammalian immortalized cell lines and certain tumor cells can maintain their telomeres in the absence of telomerase by ALT. ALT cells are characterized by a great heterogeneity in telomere length and by the presence of ALT-associated PML nuclear bodies that contain extrachromosomal telomeric DNA, telomere-specific proteins and proteins involved in DNA recombination and replication (reviewed in Henson et al., 2002). Telomeres are unique nucleoprotein structures at chromosome ends that protect chromosomes from degradation and end-to-end fusion (Chan and Blackburn, 2004). The linkage between telomere length, aging, and immortalization has been well demonstrated in animal cells. Hence, while cellular/replicative senescence is induced by telomere shortening (Sharpless and DePinho, 2004; Ben-Porath and Weinberg, 2004), ectopic expression of telomerase prevents senescence and extends the proliferative life-span of cells (Simonsen et al., 2002). In plants too, cell proliferation is commonly associated with a significant increase in telomerase activity and telomere length (Fajkus et al., 1998; Kilian et al., 1995).

So, what is the biological significance of telomerase-independent telomere lengthening that occurs in dedifferentiating cells. One possible explanation is that the increase in telomere length might be advantageous over short telomeres in reentering of Arabidopsis cells into the cell cycle. Indeed, telomere dysfunction was associated with defects in liver regeneration (Rudolph et al., 2000) and the analysis of liver regeneration after partial hepatectomy in telomerase-deficient mice shows that cells with critically short telomeres are no longer able to reenter the cell cycle (Satyanarayana et al., 2003). However, the analysis of Arabidopsis cells having short telomeres [telomerase reverse transcriptase *(tert)* mutant] showed inverse correlation between telomere length and division rate, that is, *tert* cells displayed accelerated cell cycling and higher callus weight compared to wild type cells. Thus, the increase in telomere length observed in dedifferentiated

Table 1
A list of genes that are upregulated in wild type (Ler ecotype) protoplasts (LP) but are silent or down-regulated in *kyp-2* protoplasts (KP)[a]

ATG	LP	KP	LP/KP	Description
At3g48650	810	3	245.55	Hypothetical protein
At3g47250	1017	13	81.07	Putative protein
At3g46110	723	12	61.53	Putative protein
At5g48570	797	33	23.93	Peptidylpropyl isomerase (FK-506-binding protein)
At3g47220	188	16	11.93	Phosphatidylinositol-4,5-biphosphate phosphodiesterase
At3g50760	318	28	11.31	Putative protein UDP-glucose
At1g69720	495	44	11.30	Heme oxygenase
At5g41700	3037	377	8.07	E2, ubiquitin-conjugating enzyme 8 (UBC8)
At3g28180	608	98	6.21	Unknown protein
At5g43430	1218	201	6.05	Electron transfer flavoprotein beta-subunit-like
At1g73260	4762	801	5.95	Putative trypsin inhibitor
At5g37230	179	33	5.39	Ubiquitin ligase type RING-H2 finger protein
At1g56300	984	194	5.08	DnaJ protein
At3g50260	3690	887	4.16	Putative protein EREBP-3 homolog
At3g26740	1939	474	4.09	Light regulated protein
At5g41740	509	130	3.90	Disease resistance protein-like
At1g61820	764	200	3.83	Beta-glucosidase
At1g78895	461	124	3.70	Expressed protein
At2g21660	1309	385	3.40	Glycine-rich RNA binding protein 7
At5g38320	533	159	3.36	Putative protein
At1g53870	1893	584	3.24	Hypothetical protein
At1g17280	1200	395	3.04	Ubiquitin-conjugating enzyme

Genes encoding components of the ubiquitin proteasome system are highlighted.
LP/KP indicates fold increase.
[a] Affymetrix microarray analysis. The indicated transcript level value is an average of two repeats.

protoplast cells does not appear to facilitate reentry into the cell cycle.

Another explanation is that this phenomenon induced by dedifferentiation signals (i.e., removal of cell wall) represents aberrant chromatin reorganization bringing telomeres close to interstitial telomeric repeats (ITRs), which under appropriate conditions can induce DNA recombination. This hypothesis is supported by the findings showing that plant and animal cells display global and extensive chromatin reorganization in the course of cellular dedifferentiation and reentry into the cell cycle (reviewed in Grafi, 2004), as well as by the finding showing association of subtelomeric regions with chromocenters in *Arabidopsis* during the transition from leaf cells to protoplasts (Avivi et al., 2004). ITRs were found in pericentric and heterochromatic regions of chromosomes of plants and mammals (Hastie and Allshire, 1989; Biessmann and Mason, 1994; Uchida et al., 2002), and are often correlated with genome instability, including the formation of recombination hotspots and chromosomal breakage (Hastie and Allshire, 1989; Bouffler, 1998; Bolzan and Bianchi, 2006). Indeed, increased somatic recombination appears to be a general stress response in plants (Lucht et al., 2002). Thus, dedifferentiation signals might induce not only epigenetic reprogramming of gene expression (Dean et al., 2003), but also genetic variation driven by DNA recombination. This complexity may explain pitfalls in nuclear cloning, that is, low efficiency and high level of embryonic lethality, and should be taken into consideration when reverting mature cells to an embryonic state for the use in regenerative medicine.

Our results revealed that histone methylation by KYP/SUVH4 plays an important role in setting up the dedifferentiated state and/or in reentering into the cell cycle, inasmuch as *kyp-2* mutant displayed a significant delay in reentering into the cell cycle, proliferation and callus formation. In addition, KYP/SUVH4 appears to participate in a molecular network underlying telomeric chromatin configuration and in DNA recombination as revealed by the phenomenon of telomerase-independent telomere lengthening. A link between histone methylation and telomere metabolism has been reported. Mutations in Suv39h1 and Suv39h2, both known to methylate H3-K9 at heterochromatic regions, resulted in abnormal increase in telomere length in cultured embryonic cells of mice (Garcia-Cao et al., 2004). KYP/SUVH4 may also control protein degradation by affecting the expression of genes involved the ubiquitin proteolytic pathway. Ubiquitination of proteins is a complex process, which involves the transfer of ubiquitin – the biochemical tag marking proteins for degradation – from an enzyme called E1 (ubiquitin activating enzyme) to another enzyme called E2 ubiquitin conjugating enzyme (UBC). A third enzyme called E3 ubiquitin ligase facilitates the transfer of ubiquitin from E2 to specific protein substrates (Hershko et al., 1983; Hershko, 1988). We found that two genes normally upregulated in wild-type protoplasts, namely, E2-UBC8 and E3 ligase encoded by At5g37230 are downregulated in *kyp2* protoplasts. Interestingly, UBC8 was shown to be active in ubiquitination in combination with various RING finger domain containing E3 ligases, including the E3 RING H2 encoded by the At5g37270 gene (Stone et al., 2005; Kraft et al., 2005), which is closely related to the At5g37230 gene product (93% amino acid sequence identity) found in the present work. Thus, KYP/SUVH4 appears to control the expression of both

the E2 and its corresponding E3 partner to bring about ubiquitination of, as yet unknown, protein substrates. Microarray data showed activation of polyubiquitin genes in protoplasts of both wild-type and *kyp-2* (Supporting data, Table 3), which is consistent with previous reports showing that the transition of tobacco leaf cells into protoplasts is accompanied by abrupt increase in ubiquitin gene expression (Jamet et al., 1990). Thus, activation of the ubiquitin proteolytic system might represent a critical point in cellular reorganization, namely, selective destruction of proteins involved in maintaining the old function of a cell and a concomitant activation of proteins that are essential for the establishment of dedifferentiation and/or cell proliferation. In plants, the ubiquitin system regulates various aspects of development including hormone signaling, embryogenesis and senescence (Moon et al., 2004). It has been implicated in cell cycle progression both in animals and plants (Kondorosi and Kondorosi, 2004; Hershko, 2005), and it was required for reentry of protoplasts into the cell cycle (Zhao et al., 2001).

Taken together, our results suggest that *de novo* methylation of H3K9 by KYP/SUVH4 HMTase is required for ALT as well as for the establishment of the dedifferentiated state and/or for inducing cells to reenter the cell cycle, at least partly, through activation of genes whose products are involved in the ubiquitin proteolytic pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.03.023.

References

Avivi, Y., Morad, V., Ben-Meir, H., Zhao, J., Kashkush, K., Tzfira, T., Citovsky, V., Grafi, G., 2004. Reorganization of specific chromosomal domains and activation of silent genes in plant cells acquiring pluripotentiality. Dev. Dyn. 230, 12–22.

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., Kouzarides, T., 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature 410, 120–124.

Bartee, L., Malagnac, F., Bender, J., 2001. Arabidopsis *cmt3* chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. Genes Dev. 15, 1753–1758.

Ben-Porath, I., Weinberg, R.A., 2004. When cells get stressed: an integrative view of cellular senescence. J. Clin. Invest. 113, 8–13.

Biessmann, H., Mason, J.M., 1994. Telomeric repeat sequences. Chromosoma 103, 154–161.

Blank, T., Trendelenburg, M., Kleinschmidt, J., 1992. Reactivation of DNA replication in erythrocyte nuclei by *Xenopus* egg extract involves energy-dependent chromatin decondensation and changes in histone phosphorylation. Exp. Cell Res. 202, 224–232.

Bolzan, A.D., Bianchi, M.S., 2006. Telomeres, interstitial telomeric repeat sequences and chromosomal aberrations. Mutat. Res. 612, 189–214.

Bouffler, S.D., 1998. Involvement of telomeric sequences in chromosomal aberrations. Mutat. Res. 404, 199–204.

Brockes, J.P., Kumar, A., 2005. Appendage regeneration in adult vertebrates and implications for regenerative medicine. Science 310, 1919–1923.

Chan, S.R., Blackburn, E.H., 2004. Telomeres and telomerase. Philos. Trans. R. Soc. Lond., B Biol. Sci. 359, 109–121.

Chiabrera, A., Hinsenkamp, M., Pilla, A.A., Ryaby, J., Ponta, D., Belmont, A., Beltrame, F., Grattarola, M., Nicolini, C., 1979. Cytofluorometry of electromagnetically controlled cell dedifferentiation. Histochem. Cytochem. 27, 375–381.

Criqui, M.C., Plesse, B., Durr, A., Marbach, J., Parmentier, Y., Jamet, E., Fleck, J., 1992. Characterization of genes expressed in mesophyll protoplasts of *Nicotiana sylvestris* before the re-initiation of the DNA replicational activity. Mech. Dev. 38, 121–132.

Dean, W., Santos, F., Reik, W., 2003. Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. Semin. Cell Dev. Biol. 14, 93–100.

Echeverri, K., Tanaka, E.M., 2002. Mechanisms of muscle dedifferentiation during regeneration. Semin. Cell Dev. Biol. 13, 353–360.

Fajkus, J., Fulneckova, J., Hulanova, M., Berkova, K., Riha, K., Matyasek, R., 1998. Plant cells express telomerase activity upon transfer to callus culture, without extensively changing telomere lengths. Mol. Gen. Genet. 260, 470–474.

Garcia-Cao, M., O'Sullivan, R., Peters, A.H., Jenuwein, T., Blasco, M.A., 2004. Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. Nat. Genet. 36, 94–99.

Gaudin, V., Libault, M., Pouteau, S., Juul, T., Zhao, G., Lefebvre, D., Grandjean, O., 2001. Mutations in like heterochromatin protein 1 affect flowering time and plant architecture in *Arabidopsis*. Development 128, 4847–4858.

Gisselsson, D., Lv, M., Tsao, S.W., Man, C., Jin, C., Hoglund, M., Kwong, Y.L., Jin, Y., 2005. Telomere-mediated mitotic disturbances in immortalized ovarian epithelial cells reproduce chromosomal losses and breakpoints from ovarian carcinoma. Genes Chromosomes Cancer 42, 22–33.

Gonda, K., Fowler, J., Katoku-Kikyo, N., Haroldson, J., Wudel, J., Kikyo, N., 2003. Reversible disassembly of somatic nucleoli by the germ cell proteins FRGY2a and FRGY2b. Nat. Cell Biol. 5, 205–210.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., Coupland, G., 1997. Polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. Nature 386, 44–51.

Grafi, G., 2004. How cells dedifferentiate: a lesson from plants. Dev. Biol. 268, 1–6.

Grewal, S.I., Moazed, D., 2003. Heterochromatin and epigenetic control of gene expression. Science 301, 798–802.

Hastie, N.D., Allshire, R.C., 1989. Human telomeres: fusion and interstitial sites. Trends Genet. 5, 326–331.

Hendrich, B., Tweedie, S., 2003. The methyl-CpG binding domain and the evolving role of DNA methylation in animals. Trends Genet. 19, 269–277.

Henson, J.D., Neumann, A.A., Yeager, T.R., Reddel, R.R., 2002. Alternative lengthening of telomeres in mammalian cells. Oncogene 21, 598–610.

Hershko, A., 1988. Ubiquitin-mediated protein degradation. J. Biol. Chem. 263, 15237–15240.

Hershko, A., 2005. The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. Cell Death Differ. 12, 1191–1197.

Hershko, A., Heller, H., Elias, S., Ciechanover, A., 1983. Components of ubiquitin–protein ligase system. Resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. 258, 8206–8214.

Jackson, J.P., Lindroth, A.M., Cao, X., Jacobsen, S.E., 2002. Control of CpNpG DNA methylation by the kryptonite histone H3 methyltransferase. Nature 416, 556–560.

Jackson, J.P., Johnson, L., Jasencakova, Z., Zhang, X., Perez-Burgos, L., Singh, P.B., Cheng, X., Schubert, I., Jenuwein, T., Jacobsen, S.E., 2004. Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*. Chromosoma 112, 308–315.

Jamet, E., Durr, A., Parmentier, Y., Criqui, M.C., Fleck, J., 1990. Is ubiquitin

involved in the dedifferentiation of higher plant cells? Cell Differ. Dev. 29, 37–46.
Jasencakova, Z., Soppe, W.J., Meister, A., Gernand, D., Turner, B.M., Schubert, I., 2003. Histone modifications in Arabidopsis—High methylation of H3 lysine 9 is dispensable for constitutive heterochromatin. Plant J. 33, 471–480.
Jeddeloh, J.A., Stokes, T.L., Richards, E.J., 1999. Maintenance of genomic methylation requires a SWI2/SNF2-like protein. Nat. Genet. 22, 94–97.
Jenuwein, T., Allis, C.D., 2001. Translating the histone code. Science 293, 1074–1080.
Johnson, L., Cao, X., Jacobsen, S.E., 2002. Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. Curr. Biol. 12, 1360–1367.
Kikyo, N., Wade, P.A., Guschin, D., Ge, H., Wolffe, A.P., 2000. Active remodeling of somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. Science 289, 2360–2362.
Kilian, A., Stiff, C., Kleinhofs, A., 1995. Barley telomeres shorten during differentiation but grow in callus culture. Proc. Natl. Acad. Sci. U. S. A. 92, 9555–9559.
Kondorosi, E., Kondorosi, A., 2004. Endoreduplication and activation of the anaphase-promoting complex during symbiotic cell development. FEBS Lett. 567, 152–157.
Konishi, M., Sugiyama, M., 2003. Genetic analysis of adventitious root formation with a novel series of temperature-sensitive mutants of *Arabidopsis thaliana*. Development 130, 5637–5647.
Kornberg, R.D., Lorch, Y., 1999. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell 98, 285–294.
Kraft, E., Stone, S.L., Ma, L., Su, N., Gao, Y., Lau, O.S., Deng, X.W., Callis, J., 2005. Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. Plant Physiol. 139, 1597–1611.
Kwon, C., Chung, I.K., 2003. Interaction of an Arabidopsis RNA-binding protein with plant single-stranded telomeric DNA modulates telomerase activity. J. Biol. Chem. 279, 12812–12818.
Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., Jenuwein, T., 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410, 116–120.
Lawrence, R.J., Earley, K., Pontes, O., Silva, M., Chen, Z.J., Neves, N., Viegas, W., Pikaard, C.S., 2004. A concerted DNA methylation/histone methylation switch regulates rRNA gene dosage control and nucleolar dominance. Mol. Cell 13, 599–609.
Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., Jacobsen, S.E., 2001. Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. Science 292, 2077–2080.
Lucht, J.M., Mauch-Mani, B., Steiner, H.Y., Metraux, J.P., Ryals, J., Hohn, B., 2002. Pathogen stress increases somatic recombination frequency in Arabidopsis. Nat. Genet. 30, 311–314.
Malagnac, F., Bartee, L., Bender, J., 2002. An Arabidopsis SET domain protein required for maintenance but not establishment of DNA methylation. EMBO J. 21, 6842–6852.
Moon, J., Parry, G., Estelle, M., 2004. The ubiquitin–proteasome pathway and plant development. Plant Cell 16, 3181–3195.
Odelberg, S.J., 2002. Inducing cellular dedifferentiation: a potential method for enhancing endogenous regeneration in mammals. Semin. Cell Dev. Biol. 13, 335–343.
Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., Jenuwein, T., 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406, 593–599.
Richards, E.J., Ausubel, F.M., 1988. Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. Cell 53, 127–136.
Riha, K., McKnight, T.D., Griffing, L.R., Shippen, D.E., 2001. Living with genome instability: plant responses to telomere dysfunction. Science 291, 1797–1800.
Rudolph, K.L., Chang, S., Millard, M., Schreiber-Agus, N., DePinho, R.A., 2000. Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. Science 287, 1253–1258.
Satyanarayana, A., Wiemann, S.U., Buer, J., Lauber, J., Dittmar, K.E., Wustefeld, T., Blasco, M.A., Manns, M.P., Rudolph, K.L., 2003. Telomere shortening impairs organ regeneration by inhibiting cell cycle re-entry of a subpopulation of cells. EMBO J. 22, 4003–4013.
Scebba, F., Bernacchia, G., De Bastiani, M., Evangelista, M., Cantoni, R.M., Cella, R., Locci, M.T., Pitto, L., 2003. Arabidopsis MBD proteins show different binding specificities and nuclear localization. Plant Mol. Biol. 53, 755–771.
Sharpless, N.E., DePinho, R.A., 2004. Telomeres, stem cells, senescence, and cancer. J. Clin. Invest. 113, 160–168.
Simonsen, J.L., Rosada, C., Serakinci, N., Justesen, J., Stenderup, K., Rattan, S.I., Jensen, T.G., Kassem, M., 2002. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. Nat. Biotechnol. 20, 592–596.
Stone, S.L., Hauksdottir, H., Troy, A., Herschleb, J., Kraft, E., Callis, J., 2005. Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis. Plant Physiol. 137, 13–30.
Takebe, I., Labib, G., Melchers, G., 1971. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften 58, 318–320.
Uchida, W., Matsunaga, S., Sugiyama, R., Kawano, S., 2002. Interstitial telomere-like repeats in the *Arabidopsis thaliana* genome. Genes Genet. Syst. 77, 63–67.
Valente, O., Tao, W., Verbelen, J-P., 1998. Auxins and cytokinins control DNA endoreduplication and deduplication in single cells of tobacco. Plant Sci. 134, 207–215.
Williams, L., Zhao, J., Morozova, N., Li, Y., Avivi, Y., Grafi, G., 2003. Chromatin reorganization accompanying cellular dedifferentiation is associated with modifications of histone H3, redistribution of HP1, and activation of E2F-target genes. Dev. Dyn. 128, 113–120.
Wilmut, I., Beaujean, N., de Sousa, P.A., Dinnyes, A., King, T.J., Paterson, L.A., Wells, D.N., Young, L.E., 2002. Somatic cell nuclear transfer. Nature 419, 583–586.
Wolffe, A.P., 1992. Chromatin: Structure and Function. Academic Press, San Diego.
Zelcer, A., Galun, E., 1976. Culture of newly isolated tobacco protoplasts: precursor incorporation into protein, RNA and DNA. Plant Sci. Lett. 7, 331–336.
Zemach, A., Li, Y., Wayburn, B., Ben-Meir, H., Kiss, V., Avivi, Y., Kalchenko, V., Jacobsen, S.E., Grafi, G., 2005. DDM1 binds Arabidopsis methyl-CpG binding domain proteins and affects their subnuclear localization. Plant Cell 17, 1549–1558.
Zhang, Y., Reinberg, D., 2001. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev. 15, 2343–2360.
Zhang, X., Mar, V., Zhou, W., Harrington, L., Robinson, M.O., 1999. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. Genes Dev. 13, 2388–2399.
Zhao, J., Morozova, N., Williams, L., Libs, L., Avivi, Y., Grafi, G., 2001. Two phases of chromatin decondensation during dedifferentiation of plant cells: Distinction between competence for cell fate switch and a commitment for S phase. J. Biol. Chem. 276, 22772–22778.